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Phylogenetic Analysis and PCR-Restriction Fragment Length Polymorphism Identification of *Campylobacter* Species Based on Partial *groEL* Gene Sequences

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The phylogeny of 12 *Campylobacter* species and reference strains of *Arcobacter butzleri* and *Helicobacter pylori* was studied based on partial 593-bp *groEL* gene sequences. The topology of the phylogenetic neighbor-joining tree based on the *groEL* gene was similar to that of the tree based on the 16S rRNA gene. However, *groEL* was found to provide a better resolution for *Campylobacter* species, with lower interspecies sequence similarities (range, 65 to 94%) compared with those for the 16S rRNA gene (range, 90 to 99%) and high intraspecies sequence similarities (range, 95 to 100%; average, 99%). A new universal reverse primer that amplifies a 517-bp fragment of the *groEL* gene was developed and used for PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of 68 strains representing 11 *Campylobacter* species as well as reference strains of *A. butzlerii* and *H. pylori*. Digestion with the AluI enzyme discriminated all *Campylobacter* species included in the study but showed more intraspecies diversity than digestion with the ApoI enzyme. A hippurate-negative variant of *Campylobacter jejuni* with a high level of *groEL* sequence similarity to both *C. jejuni* (96%) and *C. coli* (94%) gave a unique AluI profile and an ApoI profile identical to those of other *C. jejuni* strains. In conclusion, *groEL* gene sequencing and PCR-RFLP analysis are recommended as valuable tools for the identification of *Campylobacter* species.

Campylobacter jejuni is a major cause of bacterial gastroenteritis in humans (8). During the last two decades new *Campylobacter* species have been described, resulting in 16 species and 6 subspecies (5, 25). The clinical importance of some of these new species is as yet unknown (18). Present selective culture methods have been developed for the specific isolation of *C. jejuni* and other thermotolerant *Campylobacter* species (3). The prevalence of the more fastidious species in human disease and the environment may thus be underestimated. The asaccharolytic nature and inertness of *Campylobacter* and related species in traditional biochemical tests makes their identification difficult. In addition, the identification of *Campylobacter* species according to phenotypic properties may result in false identifications, as many species include strains that give atypical results in some key phenotypic tests (24, 28). For example, *C. jejuni* strains that lack the ability to hydrolyze hippurate have been described (37). To further understand the epidemiology and impact of *Campylobacter* species on public and animal health, better isolation and identification methods are required.

Various molecular DNA-based methods for the identification of *Campylobacter* species have been developed. These methods typically require the use of several species-specific PCR primers, hybridization probes, or multiple restriction enzymes and are usually not designed to differentiate all known species simultaneously. 16S rRNA gene sequencing and PCR-restriction fragment length polymorphism (PCR-RFLP) analysis have been described for *Campylobacter* species identification (2, 9, 22), but these methods do not differentiate between *C. jejuni* and *C. coli*. PCR-RFLP analysis of the 23S rRNA gene with two restriction enzymes is able to discriminate between *Campylobacter* species, but interpretation of the results is complicated by intervening sequences (11). More recently, amplified fragment length polymorphism fingerprinting has proven to be useful for *Campylobacter* species identification (4, 27), but the method is laborious and expensive. Whole-genome DNA-DNA hybridization analysis allows species identification, but the method is not suitable for routine use (34).

The *groEL* gene, which encodes a 60-kDa subunit (known as GroEL, 60-kDa chaperonin, and heat shock protein 60) of a complex that assists with the three-dimensional folding of bacterial proteins (7), has the potential to serve as a general phylogenetic marker because of its ubiquity and conservation in nature (32). Studies on the suitability of a fragment from a conserved region of the *groEL* gene for phylogenetic analyses and identification of species of the genera *Bifidobacterium* (13), *Helicobacter* (23), *Rickettsia* (20), *Staphylococcus* and *Macrococcus* (16), and *Vibrio* (17), among others, have been published. These studies have shown that, despite the conserved nature of the *groEL* gene, the level of interspecies *groEL* sequence variation is greater than that of the 16S rRNA gene, providing better resolution for species classification. Recently, partial *groEL* sequences from reference strains of *C. jejuni*, *C. coli*, and *C. lari* have produced similar results (39). Dot blot hybridization and PCR-RFLP analysis with AluI enzyme digestion of the partial *groEL* gene amplicon were also evaluated for the identification of *C. jejuni*, *C. coli*, and *C. fetus* subsp. *intestinalis*, with species-specific results (39).

In the present study, we cloned and sequenced 593 bp of the

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groEL gene from strains representing 11 *Campylobacter* species and a reference strain of *Arcobacter butzleri*. A new degenerate reverse primer applicable for direct sequencing was designed for specific amplification of 517 bp of the gene. PCR-RFLPs with AluI and ApoI digestion of the partial *groEL* gene amplicon were assessed by using previously characterized human and animal *Campylobacter* isolates.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. *Campylobacter* strains isolated in Finland were characterized by microscopy (Gram stain, motility); for catalase and oxidase activity, hippurate and indoxyl acetate hydrolysis, nitrate reduction, and H_2S production in triple sugar iron agar; and with the API Campy system (bioMérieux sa, Marcy l'Etoile, France), as appropriate. *C. helveticus* and *C. upsaliensis* isolates were identified by species-specific PCR (19).

Human isolate 6871, tentatively identified as *C. coli*, was further characterized by hippuricase PCR (21); 16S rRNA gene sequencing with primers 27f, 518r, 536f, 1054r, 1073f, and 1492r (10, 26); 23S rRNA gene PCR-RFLP analysis (11); and dot blot hybridization (12). Hybridization was performed with genomic DNA isolated from *C. jejuni* reference strain NCTC 11168 and labeled with digoxigenin-11-dUTP (DIG-High Prime; Roche Diagnostics GmbH, Mannheim, Germany) as the probe. Genomic DNAs from *C. coli* CCUG 11283 and *C. jejuni* NCTC 11168 were used as references for evaluation of the degree of hybridization. The hybridizations were carried out at 58°C.

Isolation of genomic DNA. Genomic DNA was isolated from cultures grown on brucella blood agar for 24 to 48 h at 37°C under microaerobic conditions, as described previously (12, 29). Alternatively, for testing of the rapid PCR-RFLP method, cell lysates were prepared by suspending a 10-μl loopful of growth in 500 μl of sterile distilled water in a microcentrifuge tube. The tubes were heated at 100°C for 10 min and subsequently cooled to 4°C. The tubes were centrifuged at 13,000 rpm (Biofuge 13, rotor 3757; Heraeus Sepatech GmbH, Osterode/Harz, Germany) for 5 min, and the supernatant was stored at −20°C.

PCR amplification and cloning of partial *groEL* gene. The partial (593-bp) *groEL* gene was amplified with the degenerate primers H60F (5′-GGN GAY GGN CAN CAN CAN GCN CAN GT-3′) and H60R (5′-TCN CCR AAN CCN GGN GCY TTN CAN GC-3′) (30) (see below for explanation of bases designated Y, N, D, or R). The amplified region corresponds to nucleotides 253 to 845 of the *groEL* gene in *C. jejuni* NCTC 11168 (GenBank accession no. AY044099). The 50-μl PCR mixture contained 200 ng of genomic DNA (or 5 μl of cell lysate), 100 μM each deoxynucleoside triphosphate, 2.5 U of *Taq* DNA polymerase (MBI Fermentas, Hanover, Md.), 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 0.08% Nonidet P-40, 1.5 mM $MgCl_2$, and 2 μM each primer. The PCR thermal cycling conditions were as described earlier (13). PCR products of the expected sizes were purified from 2% NuSieve GTG low-melting-point agarose gels (BioWhittaker Molecular Applications, Rockland, Maine) with a QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany), cloned into pGEM-T Easy vector (Promega, Madison, Wis.), and transformed into competent JM109 *Escherichia coli* cells (Promega). The transformants were selected with ampicillin, and recombinants were selected by blue-white differentiation. Plasmids were isolated from several clones with a Qiagen Plasmid Mini kit. To check for the presence of the correct 593-bp insert, plasmids were digested with EcoRI and the restriction products were separated on 1% agarose gels.

DNA sequencing. Nucleotide sequencing of three clones per strain was performed by automated cycle sequencing with Big Dye terminators (ABI 377XL; PE Applied Biosystems, Foster City, Calif.) and primers RP (reverse primer) and UP (universal primer M13-20).

Sequence analysis. The partial *groEL* gene sequences determined in this study and those available in public databases (Table 1) were analyzed with Bionumerics software (version 3.5; Applied Maths BVBA, Sint-Martens-Latem, Belgium). Distances were calculated by using all 541 bases (with the omission primer sequences). After pairwise and multiple-sequence alignments, a phylogenetic tree was constructed by the neighbor-joining method (31). Distances were corrected for multiple base changes by the method of Jukes and Cantor (14). The topology of the tree was evaluated by 1,000 trials of bootstrap analysis.

The *groEL* nucleotide sequences were translated to the corresponding amino acid sequences with the Transeq program (EMBOSS [European Molecular Biology Open Software Suite]). The resulting sequences were aligned, and a neighbor-joining tree was calculated with the ClustalW program. The phylogenetic tree based on the amino acid sequences was drawn with the TreeView program (version 1.6.6; Division of Environmental and Evolutionary Biology, University of Glasgow [http://taxonomy.zoology.gla.ac.uk/rod/rod.html]).

Design of new reverse PCR primer. A new degenerate reverse PCR primer was developed for the conserved protein sequence AEDIEGE. The nucleotide sequence of the new reverse primer H60R1 was 5′-C YTC NCC YTC DAT RTC YTC NGC-3′ (517-bp product, corresponding to nucleotides 253 to 769 of the *groEL* gene in *C. jejuni* NCTC 11168, where Y is C or T; N is G, A, T, or C; D is G, T, or A; and R is A or G). The new primer was developed to have a melting temperature closer to that of the forward primer and to increase the specificity of the PCR. The melting temperature, primer-dimer, and secondary structure formation were checked by using the Sigma-Genosys basic oligonucleotide calculator (http://www.sigma-genosys.co.uk/oligos/frameset.html).

PCR-RFLP typing of *Campylobacter* isolates. On the basis of computational restriction fragment analysis of the partial *groEL* gene sequences with the Restriction Mapper program (version 3; http://www.restrictionmapper.org/), the AluI and ApoI restriction enzymes were chosen, as they were expected to yield sufficient fragment numbers of sufficient sizes to produce species-specific restriction profiles. Digestions were performed with 20 μl of the PCR products in a total volume of 25 μl with 5 U of AluI or 4 U of ApoI (New England Biolabs Inc., Beverly, Mass.). The resulting fragments were separated electrophoretically (90 V for 3 h) in 4% MetaPhor agarose gels in 1× Tris-acetate-EDTA buffer. The gels were stained with ethidium bromide and visualized under UV light. The resulting patterns were analyzed with BioNumerics software.

Nucleotide sequence accession numbers. The partial *groEL* and 16S rRNA gene sequences obtained in this study were deposited in GenBank. The accession numbers are shown in Table 1. For comparison, published *groEL* and 16S rRNA gene sequences were downloaded from GenBank (Table 1).

RESULTS

Phylogenetic analysis of the partial *groEL* gene sequences compared with 16S rRNA gene sequences. The neighbor-joining tree constructed from the partial *groEL* gene sequences is shown in Fig. 1. The major topology of the tree based on the partial *groEL* gene sequences was similar to that based on the 16S rRNA gene sequences. The more detailed similarity analysis of the partial *groEL* and 16S rRNA gene sequences among *Campylobacter* species, *A. butzleri*, and *Helicobacter pylori* is shown in Table 2. The similarities of the *groEL* sequence of *Campylobacter* species to those of *A. butzleri* strain CCUG 10373 and *H. pylori* strain 26695 were in the range of 51 to 73% and 56 to 67%, respectively, whereas the 16S rRNA gene sequence similarities were 83 to 85% and 81 to 83%, respectively (Table 2). The interspecies *groEL* sequence similarities between *Campylobacter* species ranged from 65% (between *C. fetus* subsp. *fetus* and *C. rectus*) to 94% (between *C. jejuni* and *C. coli*) and 100% (between *C. hyoilei* and *C. coli*) (Fig. 1). *C. upsaliensis* and *C. helveticus* formed a separate branch with high bootstrap support, as did *C. coli* and *C. jejuni* as well as *C. lanienae*, *C. fetus* subsp. *fetus*, and both *C. hyointestinalis* subspecies (Fig. 1). The partial *groEL* gene sequences of the type strains of *C. upsaliensis* and *C. helveticus* showed 91% similarity, whereas the 16S rRNA gene sequences showed 98% similarity (Table 2). The 16S rRNA gene sequence similarity between *C. helveticus* and *C. jejuni* was high (97%), but the partial *groEL* gene similarity was only 84%. The *groEL* sequence similarity of *C. coli* and *C. jejuni* was 91%, whereas the 16S rRNA gene sequence similarity was 98%. *C. lari* showed 86 and 87% *groEL* gene sequence similarity to *C. jejuni* and *C. coli*, respectively, whereas the 16S rRNA gene sequence similarities were 98 and 97%, respectively. Similarly, the similarity of the *C. lanienae groEL* gene sequence to those of *C. hyointestinalis* subsp. *hyointestinalis* and *C. hyointestinalis* subsp. *lawsonii* was 81%, whereas the 16S rRNA gene sequence similarities were 97 and 98%, respectively. The *groEL* sequence similarity be-

TABLE 1. Sources, PCR-RFLP profiles, and sequence accession numbers of the strains included in the study

Species and strain	Source	AluI profile	ApoI profile	GenBank accession no.[a]	
				groEL gene	16S rRNA gene
A. butzleri CCUG 10373	Reference strain	11	9	**AY628390**	L14626
C. coli					
CCUG 11283^T	Type strain	10a	8	**AY628391**	M59073, L04312
NCTC 11353	Reference strain			AY044098 (39)[b]	
4195	Human, Finland	10a	8	**AY628392**	
S110R	Porcine, Finland	10c	6		
S120R	Porcine, Finland	10b	8		
S127R	Porcine, Finland	10c	6		
S139R	Porcine, Finland	10a	8		
S140R	Porcine, Finland	10a	8		
S149R	Porcine, Finland	10c	6		
S152R	Porcine, Finland	10a	8		
S157R	Porcine, Finland	10a	8		
S161Ra	Porcine, Finland	10c	6		
S161Rb	Porcine, Finland	10a	8		
S163R	Porcine, Finland	10a	8		
S170R	Porcine, Finland	10c	6		
S173R	Porcine, Finland	10a	8		
S176R	Porcine, Finland	10c	6		
S178R	Porcine, Finland	10a	8		
S183R	Porcine, Finland	10a	8		
S190R	Porcine, Finland	10a	8		
S210R	Porcine, Finland	10b	8		
C. fetus subsp. *fetus*					
CCUG 44789	Reference strain	2	2	**AY628393**	
976	Human, Finland	2	2		
13014	Human, Finland	2	2		
14865	Human, Finland	2	2		
C. helveticus					
CCUG 30682^T	Type strain	6	4	**AY628394**	U03022
KI460	Feline, Finland	6	4	**AY628395**	
C. hyoilei CCUG 33450^T	Type strain	10a	8	**AY628396**	
C. hyointestinalis subsp. *hyointestinalis*					
CCUG 14169^T	Type strain	3	2	**AY628397**	AF097689
s826	Bovine, Finland	3	2		
r1385	Bovine, Finland	3	2		
PO0	Reindeer, Finland	3	2		
PO57	Reindeer, Finland	3	2		
PO885	Reindeer, Finland	3	2		
C. hyointestinalis subsp. *lawsonii* CCUG 34538^T	Type strain	3	2	**AY628398**	AF097685
C. jejuni					
NCTC 11168	Reference strain	8a	6	AY044099 (39)[b]	AL111168
ATCC 43429	Reference strain			AF461064	
ATCC 43430	Reference strain			AF461533	
ATCC 43432	Reference strain			AF461534	
ATCC 43438	Reference strain			AF461535	
ATCC 43446	Reference strain			AF461536	
ATCC 43456	Reference strain			AF461537	
r774	Bovine, Finland	8a	6		
r859	Bovine, Finland	8a	6		
4117	Human, Finland	8a	6	**AY628399**	
6871	Human, Finland	8b	6	**AY628400**	**AY628389**
7635	Human, Finland	8a	6	**AY628401**	
71497	Human, Finland	8a	6		
71514	Human, Finland	8a	6		
71519	Human, Finland	8a	6		
71543	Human, Finland	8a	6		
71594	Human, Finland	8a	6		
71597	Human, Finland	8a	6		
71609	Human, Finland	8a	6		
71612	Human, Finland	8a	6		
71627	Human, Finland	8a	6		
71637	Human, Finland	8a	6		
71643	Human, Finland	8a	6		

Continued on following page

TABLE 1—*Continued*

Species and strain	Source	AluI profile	ApoI profile	GenBank accession no.[a]	
				groEL gene	16S rRNA gene
71652	Human, Finland	8a	6		
71655	Human, Finland	8a	6		
C. lanienae CCUG 44467^T	Type strain	1	1	**AY628402**	AF043425
C. lari					
CCUG 23947^T	Type strain	7a	5	AY044100 (39)[b]	L04316
L33	Seagull, Finland	7b	5	**AY628403**	
L71	Seagull, Finland	7b	5	**AY628404**	
L133	Seagull, Finland	7b	5	**AY628405**	
C. mucosalis CCUG 6822^T	Type strain	5	4	**AY628406**	L06978
C. rectus ATCC 33238^T	Type strain			AB071388 (36)[b]	L04317
C. sputorum subsp. *bubulus* CCUG 11289^T	Type strain	12	10	**AY628407**	
C. sputorum biovar fecalis CCUG 12015	Reference strain	12	10		
C. sputorum biovar sputorum CCUG 9728^T	Type strain	12	10	**AY628408**	X67775
C. upsaliensis					
CCUG 14913^T	Type strain	9a	7	**AY628409**	L14628
485 C/S	Canine, Finland	9c	7		
D86/VELL	Canine, Finland	9b	7		
KI492	Feline, Finland	9c	7		
KO797	Canine, Finland	9a	7	**AY628410**	
KO798	Canine, Finland	9d	7		
H. pylori 26695	Reference strain	4	3	AE000523	NC_000915

[a] Sequences obtained in this study are indicated in boldface.
[b] Reference for original citation of sequence data.

tween *C. fetus* and *C. hyointestinalis* subsp. *hyointestinalis* and *C. hyointestinalis* subsp. *lawsonii* was 85%.

Strain 6871 had a higher level of *groEL* sequence similarity to the *C. jejuni* strains (94.6 to 96%) than to the *C. coli* strains (92.6 to 93.6%). The strain was negative by the hippurate hydrolysis test and hippuricase gene PCR. The partial (1,470-bp) 16S rRNA gene sequence of strain 6871 showed the highest degree of similarity to *C. jejuni* strains ATCC 43431 (99.9%) and NCTC 11168 (99.7%) in a search with the BLAST algorithm. The level of sequence similarity to the type strain of *C. coli* CCUG 11283 was, however, also high (98.0%). PCR-RFLP analysis of the 23S rRNA gene resulted in a *C. jejuni*-specific profile for strain 6871. Dot blot hybridization of the whole genomic DNA of strain 6871 gave a more intense signal with *C. jejuni* than with the *C. coli* reference strain.

The intraspecies sequence similarities for *C. coli* ranged from 99 to 100%, those for *C. jejuni* ranged from 95% (98% when the hippurate-negative *C. jejuni* strain 6871 was excluded) to 100%, and those for *C. lari* ranged from 99 to 100%. The intraspecies sequence similarities between the two strains of *C. helveticus* and *C. upsaliensis* were 99.4 and 97.6%. The sequence similarity between *C. hyointestinalis* subsp. *hyointestinalis* and *C. hyointestinalis* subsp. *lawsonii* was 97.9%. The sequence similarity between *C. sputorum* biovar sputorum and *C. sputorum* subsp. *bubulus* was 99.8%.

The neighbor-joining tree based on the deduced partial GroEL amino acid sequences (180 amino acids) is shown in Fig. 2. Hippurate-negative *C. jejuni* strain 6871 had 100% amino acid similarity to *C. jejuni* strain 4117. The amino acid similarities between *C. upsaliensis* (two strains) and *C. hyointestinalis* subsp. *hyointestinalis* and *C. hyointestinalis* subsp. *lawsonii* were 100%. The intraspecies amino acid similarities of the other species were equal to the nucleotide sequence similarities, whereas the interspecies amino acid similarities were higher than the nucleotide sequence similarities, ranging from 84% (between *C. hyointestinalis* and *C. lari*) to 99% (between *C. jejuni* and *C. coli*) and 100% (between *C. hyoilei* and *C. coli*). *A. butzleri* strain CCUG 10373 and *H. pylori* strain 26695 had GroEL amino acid sequence similarities to the *Campylobacter* species GroEL amino acid sequences that ranged from 70 to 75% and 77 to 81%, respectively.

PCR-RFLP identification of *Campylobacter* species based on partial 517-bp *groEL* gene amplicons. The results of the computational restriction fragment length analysis with the AluI and ApoI enzymes are shown in Table 3. According to these results, all *Campylobacter* species studied had species-specific AluI digestion patterns and all but *C. helveticus* and *C. mucosalis* and *C. rectus* and *H. pylori* had species-specific ApoI digestion patterns. The AluI and ApoI digestion patterns of the 517-bp *groEL* gene amplicons of representative *Campylobacter* strains from 11 species and 3 subspecies or biovars, as well as reference strains of *A. butzleri* and *H. pylori*, are shown in Fig. 3. Table 1 lists the profiles obtained for the remaining strains.

Visually, 19 and 10 separate profiles were obtained by AluI and ApoI digestion, respectively. AluI digests alone discriminated among all species included in the study. For *C. coli*, *C. jejuni*, *C. lari*, and *C. upsaliensis*, three, two, two, and four

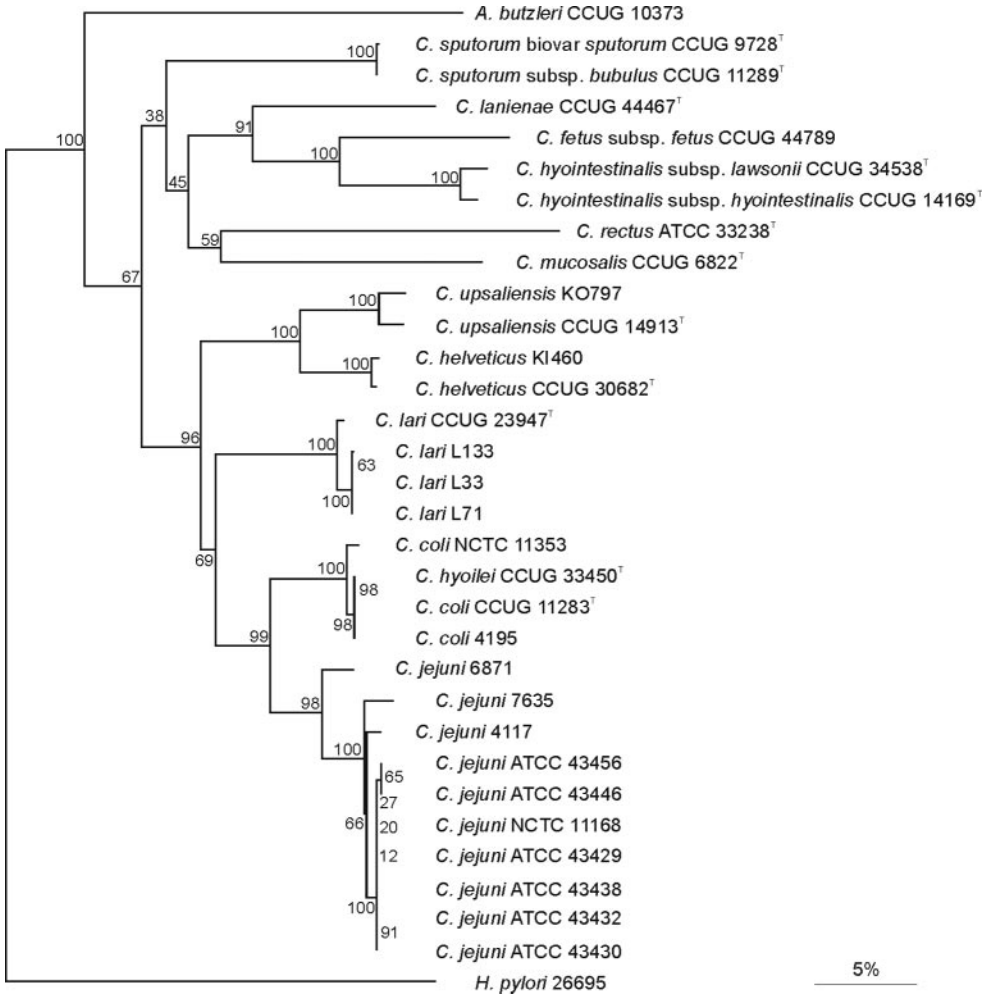

FIG. 1. Neighbor-joining tree based on partial 541-bp *groEL* sequences. The tree was rooted with *H. pylori.* The number at each branch point represents the percentage of bootstrap support calculated from 1,000 trees. The scale bar represents the sequence divergence.

separate AluI restriction profiles, respectively, were observed. Hippurate-negative strain *C. jejuni* 6871 was the only strain to yield AluI restriction profile 8b. Two ApoI restriction patterns were observed for *C. coli*. The ApoI restriction patterns could not differentiate between *C. helveticus* and *C. mucosalis*, *C. hyointestinalis* and *C. fetus*, or the less common ApoI profile 6 of *C. coli* (observed in 6 of 21 cases) and *C. jejuni*.

An atypical restriction profile for *C. coli* isolate S161R from

TABLE 2. Similarity analysis of partial *groEL* and 16S rRNA sequences among *Campylobacter* species, *A. butzleri*, and *H. pylori*

Species	% Similarity[a]													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. *A. butzleri* CCUG 10373		84	84	84	83	85	85	84	84	85	84	83	84	83
2. *C. coli* CCUG 11283^T	69		96	95	96	98	99	96	97	94	93	91	95	82
3. *C. helveticus* CCUG 30682^T	66	84		93	94	97	96	94	96	92	91	90	98	83
4. *C. hyointestinalis* subsp. *hyointestinalis* CCUG 14169^T	63	72	71		96	94	94	97	94	95	93	91	92	81
5. *C. hyointestinalis* subsp. *lawsonii* CCUG 34538^T	63	71	71	98		95	95	98	95	94	93	92	93	82
6. *C. jejuni* NCTC 11168	68	91	84	73	72		100	95	98	93	92	91	96	83
7. *C. jejuni* strain 6871	68	94	84	72	71	96		95	98	93	92	91	96	83
8. *C. lanienae* CCUG 44467^T	64	75	73	81	81	76	76		95	95	92	91	93	82
9. *C. lari* CCUG 23947^T	73	87	84	72	72	86	86	73		93	92	90	95	83
10. *C. mucosalis* CCUG 6822^T	61	71	73	75	75	73	73	75	72		94	92	92	81
11. *C. rectus* ATCC 33238^T	51	68	69	66	67	71	71	70	65	71		94	91	81
12. *C. sputorum* biovar sputorum CCUG 9728^T	70	79	76	79	80	77	78	76	80	75	69		90	81
13. *C. upsaliensis* CCUG 14913^T	66	83	91	72	71	82	82	73	82	71	69	76		83
14. *H. pylori* 26695	56	63	63	61	60	61	61	63	67	59	56	59	65	

[a] The values above the diagonal indicate the percent similarities of the partial (94%) 16S rRNA gene sequences, and the values below the diagonal indicate the percent similarities of the partial (33%) *groEL* gene sequences. The numbers in the subheads correspond to the numbers for the species in the leftmost column.

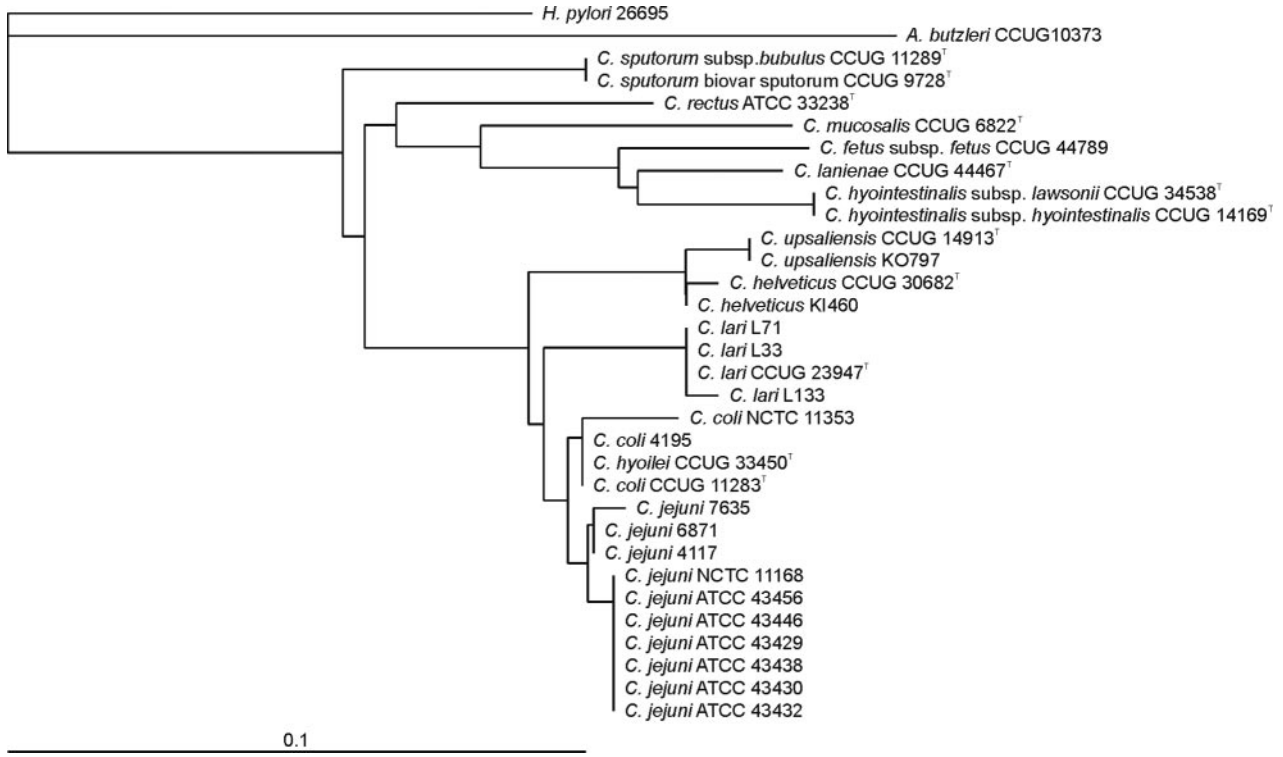

FIG. 2. Neighbor-joining tree based on the deduced partial 180-amino-acid GroEL sequences. The tree was rooted with *H. pylori.* The scale bar represents the sequence divergence.

a pig was observed. After the subculture of separate colonies, this isolate was subsequently identified as a coculture of two *C. coli* strains, strains S161Ra and S161Rb, representing two separate AluI-ApoI profiles, 10a-8 and 10c-6, respectively.

DISCUSSION

The major topology of the phylogenetic neighbor-joining tree constructed from the partial *groEL* gene sequences was similar to that constructed from the 16S rRNA gene sequences. However, *groEL* was found to provide better resolution for *Campylobacter* species, with lower interspecies sequence similarities (range, 65 to 94%) compared to that obtained with the 16S rRNA gene (range, 90 to 99%). The intraspecies *groEL* gene sequence similarities (range, 95 to 100%; average, 99%) were high compared to the interspecies sequence similarities, and strains representing a distinct

TABLE 3. Computational restriction fragment length analysis of the partial *groEL* gene product obtained with the AluI and ApoI enzymes[a]

Taxon	Profile (fragment sizes [bp])	
	AluI	ApoI
C. lanienae CCUG 44467^T	1 (149, 129, 70, 65)	1 (320, 109, 87)
C. fetus subsp. *fetus* CCUG 44789	2 (278, 147)	2 (352, 109)
C. hyointestinalis subsp. *hyointestinalis* CCUG 14169^T	3 (109, 90, 84, 65)	2 (320, 109)
C. hyointestinalis subsp. *lawsonii* CCUG 34538^T	3 (109, 90, 84, 65)	2 (320, 109)
H. pylori 26695	4 (233, 148, 135)	3 (no cut sites)
C. mucosalis CCUG 6822^T	5 (168, 129, 84, 65)	4 (228, 201)
C. helveticus CCUG 30682^T	6 (252, 129, 65)	4 (228, 201)
C. lari CCUG 23947^T	7a (252, 114, 81)	5 (239, 201)
C. jejuni NCTC 11168	8a (266, 129, 81)	6 (222, 207)
C. jejuni strain 6871	8b (266, 129, 90)	6 (222, 207)
C. upsaliensis CCUG 14913^T	9a (155, 129, 112, 88)	7 (260, 201)
C. coli CCUG 11283^T	10a (386, 70)	8 (207, 201)
C. hyoilei CCUG 33450^T	10a (386, 70)	8 (207, 201)
A. butzleri CCUG 10373	11 (284, 112, 102)	9 (321, 201)
C. sputorum subsp. *bubulus* CCUG 11289^T	12 (109, 97, 84, 71, 60)	10 (228, 201, 87)
C. sputorum biovar sputorum CCUG 9728^T	12 (109, 97, 84, 71, 60)	10 (228, 201, 87)
C. rectus ATCC 33238^T	ND[b] (252, 129, 121)	ND (no cut sites)

[a] The *groEL* gene product was obtained by PCR with primers H60F and H60R1. Only restriction fragments equal to or larger than 60 bp were included.
[b] ND, not determined; the results are from computational RFLP analysis.

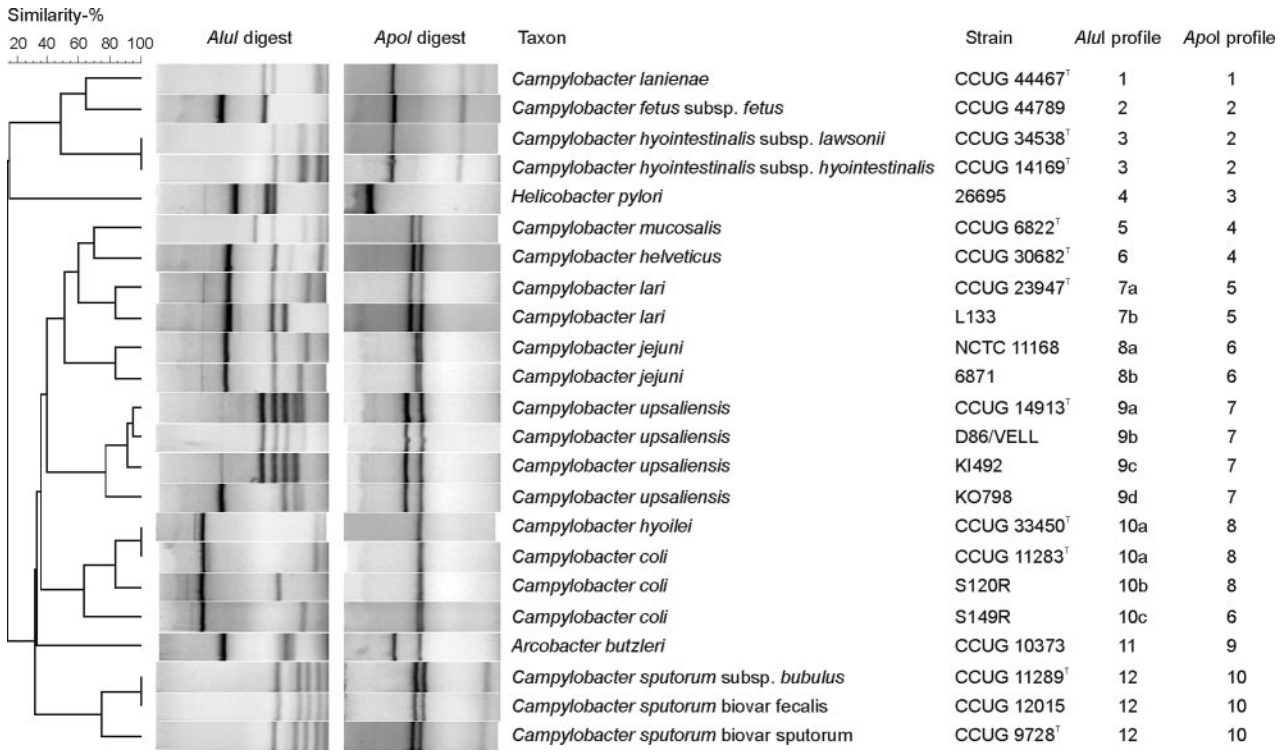

FIG. 3. RFLP analysis of the partial 517-bp *groEL* gene amplicon digested with AluI and ApoI. The combined dendrogram was derived with BioNumerics software by using the unweighted pair group method with arithmetic averages and the Dice coefficient with 1% optimization and tolerance.

Campylobacter species or two subspecies clustered tightly together. These results suggest an added value of the *groEL* gene sequence over the 16S rRNA gene sequence for *Campylobacter* species identification.

The most important phenotypic test used to differentiate between *C. jejuni* and *C. coli* is hydrolysis of hippurate, which relies on the activity of the hippuricase enzyme characteristic for *C. jejuni*. Strain 6871 was identified as an atypical (hippurate-negative) *C. jejuni* strain, according to PCR-RFLP analysis of the 23S rRNA gene and dot blot hybridization. Similar hippurate-negative *C. jejuni* strains have been shown to be quite uncommon (33, 37). In one study, 1.6% of human and poultry strains were reported to be hippurate negative according to the results of quantitative DNA hybridizations and hippurate hydrolysis experiments (37). In another study, some *C. jejuni* strains (less than 1%) did not produce a PCR product for the hippuricase gene (33). Hippurate-negative strains producing a hippuricase PCR product smaller than expected have also been reported (6, 35). Strain 6871 had a unique AluI profile (profile 8b), whereas all other *C. jejuni* strains showed identical profiles. Strain 6871 also showed only 94.6 to 96% *groEL* sequence similarity with *C. jejuni*, whereas the intraspecies sequence similarities among the other strains ranged from 98 to 100%. Hippurate-negative *C. jejuni* strains may represent a distinct clonal lineage of *C. jejuni*, as proposed previously (15).

Strains first described as a distinct species, *C. hyoilei* (1), have subsequently been proposed to represent a variant of *C. coli*, according to indistinguishable results by 66 phenotypic tests and a high DNA-DNA hybridization level (38). The type strains of these two species showed 100% *groEL* sequence similarity. *C. sputorum* subsp. *bubulus* has been reclassified as *C. sputorum* biovar sputorum (26). The two type strains of *C. sputorum* studied had a high *groEL* sequence similarity (99.8%), supporting earlier findings.

The new degenerate reverse primer H60R1 yielded specific amplification of the desired 517-bp fragment of the *groEL* gene and was used as the basis for the PCR-RFLP assay. It was also successfully tested for use for the direct sequencing of the partial *groEL* gene. With direct sequencing the cumbersome cloning step may be omitted, and sequence data for novel *Campylobacter* species and/or isolates can easily be obtained and evaluated against known species. Because the sequence divergence of the *groEL* gene is much higher, only one primer pair is required for species identification, whereas three primer pairs (or four primer pairs for specific variable regions) are required for 16S rRNA gene sequencing (9). Another advantage of the partial *groEL* gene is that it has not been shown to contain intervening sequences, which complicate the alignment and comparison of 16S and 23S rRNA gene sequences and the interpretation of the results of PCR and PCR-RFLP assays based on these genes (25).

AluI PCR-RFLP analysis was found to give species-specific discrimination. In contrast to an earlier report of a study that used a limited number of *C. coli*, *C. jejuni*, and *C. fetus* subsp. *intestinalis* strains (39), more than one AluI profile was seen for the *C. coli*, *C. jejuni*, *C. lari*, and *C. upsaliensis* strains tested in our study. The profiles of a single species differed by two to three fragments. Due to genetic drift, which results in new PCR-RFLP profiles, the use of two restriction enzymes may be recommended. ApoI produced two profiles only for *C. coli*

strains, one of which was identical to *C. jejuni* profile 6. ApoI did not discriminate between *C. helveticus* and *C. mucosalis*, *C. rectus* and *H. pylori*, or *C. hyointestinalis* and *C. fetus*; yet all of these species could be easily differentiated by their AluI profiles. In contrast, AluI profile 7a of *C. lari* was highly similar to profile 8a of *C. jejuni*, but these species could be distinguished by use of computer-assisted analysis and the respective ApoI profiles. Thus, only one or two restriction enzymes are needed to identify *Campylobacter* isolates to the species level, whereas two (22) and six (2) restriction enzymes are needed for the 16S rRNA gene-based PCR-RFLP methods, which additionally lack the ability to differentiate between *C. jejuni* and *C. coli*.

In conclusion, our results show that partial *groEL* gene sequencing and PCR-RFLP analysis are more suitable and simple methods for *Campylobacter* species-specific identification than the respective analyses of the 16S rRNA gene. Taxonomic studies of novel *Campylobacter* species are likely to benefit from the *groEL* gene sequence information. Further studies are needed to confirm the numbers of species-specific AluI and ApoI profiles and the utility of the PCR-RFLP assay.

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